ELSEVIER

FEMS Immunology and Medical Microbiology 43 (2005) 81–89

FEMS Immunology and Medical Microbiology

www.fems-microbiology.org

In vitro activation of chicken leukocytes and in vivo protection against *Salmonella enteritidis* organ invasion and peritoneal *S. enteritidis* infection-induced mortality in neonatal chickens by immunostimulatory CpG oligodeoxynucleotide

Haiqi He [a,*], Virginia K. Lowry [b], Christina L. Swaggerty [a], Pamela J. Ferro [c], Michael H. Kogut [a]

[a] *Southern Plains Agricultural Research Center, USDA-ARS, 2881 F&B Road, College Station, TX 77845, USA*
[b] *Department of Veterinary Anatomy and Public Health, Texas A&M University, College of Veterinary Medicine, College Station, TX 77843, USA*
[c] *Texas Veterinary Medical Diagnostic Laboratory, Texas A&M University, College Station, TX 77843, USA*

Received 28 May 2004; received in revised form 22 July 2004; accepted 23 July 2004

First published online 12 August 2004

Abstract

Unmethylated CpG oligodinucleotides (CpG-ODN) flanked by specific bases found in bacterial DNA are known to stimulate innate immune responses. In this study, synthetic CpG-ODNs were evaluated for their in vitro stimulation of leukocyte and in vivo protection against *Salmonella enteritidis* (SE) in neonatal chickens. Our studies showed that CpG-ODN stimulated bactericidal activities, releasing granules (degranulation) and generating reactive oxygen species (oxidative burst), in chicken heterophils and up regulated nitric oxide production in chicken peripheral blood monocytes. When day-old chickens were given (i.p.) synthetic CpG-ODNs followed by oral challenge of SE, a significant reduction ($p < 0.05$) of organ invasion by SE was observed in chickens pretreated with CpG-ODN containing the immunostimulatory GTCGTT motif. This CpG-OND also significantly reduced mortality of chickens with acute peritoneal infection of SE. Our study provides evidence that immunostimulatory CpG-ODN stimulated innate immune activities and enhanced the resistance to infectious pathogens in neonatal chickens.

Keywords: CpG-ODN; *Salmonella enteritidis*; Infection; Innate immunity; Leukocytes; Chicken

1. Introduction

DNA from bacteria is a powerful stimulator of the innate immune system [1]. In contrast to vertebrate DNA, bacterial DNA contains relatively abundant unmethylated CpG dinucleotides [2]. These unmethylated CpG dinucleotides, in particular base contents (CpG motif), are now known to attribute the immune stimulatory activities of the bacterial DNA [3]. Similar immune stimulatory activities have been demonstrated using synthetic CpG oligodeoxynucleotides (CpG-ODN) [3,4]. It has been well documented that bacterial DNA and synthetic CpG-ODN stimulate B-lymphocyte and innate immune cells such as macrophages, dendritic cells and natural killer cells from mammalian species activation and secretion of cytokines, including interleukin-1β

* Corresponding author. Tel.: +1 979 260 3771; fax: +1 979 260 9332.
E-mail address: he@ffsru.tamu.edu (H. He).

0928-8244/$22.00
doi:10.1016/j.femsim.2004.07.009

(IL-1β), IL-6, IL-12, IL-18, tumor necrosis factor-α (TNF-α), interferon-α (IFN-α), and IFN-γ [1]. Toll-like receptor 9 (TLR9) has been demonstrated to mediate CpG-ODN immune stimulatory activity in murine and human immune cells [5,6]. Recent studies have demonstrated the immune stimulatory activities of CpG-ODN in other vertebrate species, such as fish [7–9] and chicken [10–14].

Host immune defense against infectious pathogens relies on both the innate and adaptive immunity. The innate immune system provides the first line defense to limit the invading pathogen at the initial stage of infection and directs the development of adaptive immune responses [15]. Stimulation of the innate immune system in newly hatched chickens is of particularly interest to the poultry industry, because young chickens are susceptible to bacterial infection such as *Salmonella* [16]. Bacterial DNA and CpG-ODN have previously been demonstrated to stimulate innate immune responses and provided protection against infections in animals. For example, pretreatment of mice with CpG-ODN provided protection against lethal challenge of bacteria *Listeria monocytogenes* [17,18], *Francisella tularensis* [18], or parasitic protozoans *Leishmania major* [19], *Plasmodium yoelii* and *P. falciparum* [20]. Recently, CpG-ODN has also been reported to protect chickens from *Escherichia coli* infection [13].

In a previous study, we have identified that CpG-ODN# 17with GTCGTT motif strongly stimulated nitric oxide (NO) production and IL-1β expression in an avian microphage cell line HD11 and CpG-ODN#1 with GACGTT motif stimulated IFN-γ expression in peripheral blood monocytes, but less NO production and no IL-1β expression in HD11 [11]. In this study, we reported in vitro activation of chicken leukocytes, heterophils and peripheral blood monocytes, and in vivo protection against *S. enteritidis* (SE) infection by synthetic immune stimulatory CpG-ODNs in newly hatched chickens.

2. Materials and methods

2.1. Synthetic ODNs and reagents

The nuclease-resistant phosphorothioate ODNs were purchased from Biosource International (Camarillo, CA, USA) and further purified by ethanol precipitation. ODNs were dissolved in sterile phosphate-buffered saline (PBS, pH 7.2) at a concentration of 1 mg/ml, heated in boiling water for 5 min and immediately chilled on ice. Unused ODN solutions were stored at −20 °C. The sequences of synthetic ODNs used in the present study were: CpG-ODN# 1, TCG ATC GAC GTT GAG GGG GG; CpG-ODN# 17, GTC GTT GTC GTT GTC GTT; and a control ODN without CpG motif (nCpG-ODN), CCA TGG CCA TGG CCA TGG [11]. All media, medium-additives, Histopaque gradients, and other reagents used in this study were purchased from Sigma (St. Louis, MO, USA). Cell culture 96-well plate was purchased from Becton Dickinson (Franklin Lakes, NJ, USA).

2.2. Animals

Leghorn chickens (Hy-Line W-36) used for experiments were obtained from a commercial hatchery (Hy-Line International, Bryan, TX, USA) on the day-of-hatch and placed on floor pens with pine shavings in a controlled and isolated environment. Chickens were provided ad libitum access to water and a balanced unmedicated corn-soybean based diet with nutrient rations met or exceeded the recommendation by the National Research Council [21].

2.3. Cell isolation

Chicken heterophils and peripheral blood mononuclear cells (PBMC) were isolated from the peripheral blood collected from two- or three-day-old chickens as described by Kogut et al. [22]. Briefly, peripheral blood from approximately 100 chicks was pooled, mixed with 1% methylcellulose (1:1 v/v), and centrifuged at 25*g* (rcf) for 15 min. The supernatant was diluted with Ca^{2+}- and Mg^{2+}-free Hanks balanced salt solution, carefully layered onto a discontinuous Histopaque gradient (specific gravity 1.077/1.119) in 50 ml conical centrifuge tubes, and centrifuged at 250*g* (rcf) for 60 min. The PBMC layer at the 1.077/supernatant interface was collected, washed, and resuspended in RPMI-1640. The heterophils, located below the Histopaque 1.077/1.119 interface, were collected, washed, and resuspended in RPMI-1640. Heterophils and PBMC were counted and kept on ice until used.

2.4. Degranulation assay

Heterophil degranulation was measured by quantifying β-glucuronidase activity [22] in culture medium following stimulation of heterophils (8×10^6/ml) with CpG-OND in the presence of 5% chicken serum for 60 min on a rocker platform in a 5% CO_2, 95% humidity, and 39 °C incubator. After incubation, the cells were pelleted by centrifugation at 10,000*g* (rcf) for 2 min at 4 °C and supernatants were collected for the assay. An aliquot of 25 μl supernatant was incubated with 50 μl of freshly prepared substrate (10 mM 4-methylumbelliferyl-β-D-glucuronide and 0.1% Triton X-100 in 0.1 M sodium acetate buffer) in a black 96-well plate for 4 h at 41 °C. The reaction was stopped by adding 200 μl of stop solution (0.05 M glycine and 5 mM EDTA; pH 10.4) to each well. Liberated 4-methylumbelliferone

was measured fluorometrically (355/460 nm) using an f_{max} fluorescence microplate reader (Molecular Devices, Sunnyvale, CA, USA).

2.5. Oxidative burst assay

Production of reactive oxygen species (ROS) by neonatal chicken heterophils during oxidative burst was measured by oxidation of DCFH-DA to fluorescent DCF as described by He et al. [23]. Briefly, chicken heterophils and PBMC (8×10^6 cells/ml in RPMI) were incubated in 2-ml microcentrifuge tubes containing 5% chicken serum, 10 μg/ml of DCFH-DA, and various concentration of CpG-ODN for 1 h at 37 °C in a 5% CO_2 and 95% humidity incubator. The aliquots of cell cultures (150 μl) were then dispensed to a black 96-well plate and the fluorescence intensity was measured (485/530 nm) using an f_{max} fluorescence microplate reader (Molecular Devices). The relative fluorescent units (RFU) were recorded at 60-min intervals for 3 h.

2.6. Monocyte culture and nitrite assay

Aliquots of 200 μl of PBMC (1×10^7 cells/ml) were dispensed to a 96-well (round-bottom plate) and incubated at room temperature for 2 h. After incubation, non-adherent cells were removed by washing twice with the culture medium (Dulbecco's Modified Eagles Medium (DMEM) containing 10% chicken serum, antibiotics (100 U penicillin/ml and 100 μg streptomycin/ml), and 1.5 mM L-glutamine). The adherent monocytes in 200 μl culture medium per well were stimulated with CpG-ODNs for 48 h at 41 °C in a 5% CO_2 and 95% humidity incubator. Nitrite produced by activated monocytes was measured by the Greiss assay [24]. Briefly, aliquot of 100 μl culture supernatant from each well was transferred to a new 96-well plate (flat-bottom) and combined with 50 μl of 1% sulfanilamide and 50 μl of 0.1% naphthylenediamine (both were prepared in 2.5% phosphoric acid solution). After 10 min incubation at room temperature, the nitrite concentration was determined by measuring optical density (OD_{595}) of each well using a SPECTRA MAX microplate reader (Molecular Devices). Sodium nitrite (Sigma) was used as a standard to determine nitrite concentrations in the cell-free medium.

2.7. Bacteria

A primary poultry isolate of *S. enteritidis* was obtained from the National Veterinary Services Laboratory, Ames, IA 50010 and approved for use in our laboratory by the USDA Animal and Plant Health Inspection Service. A carbonicillin–novobiocin (C–N) resistant isolate was selected and stored in 75% trypticase soy broth (TSB) + 25% sterile glycerol in aliquots of 1×10^9 colony forming units (CFU) at −70 °C until used. The TSB used to culture the resistant isolate contained 100 μg/ml C and 25 μg/ml N to inhibit the growth of other bacteria. The SE for oral challenge was cultured over night at 41 °C, washed, and resuspended in the sterile PBS (pH 7.2) at a concentration of 10^8 CFU/ml. The viable cell concentration of SE was determined by colony counts on brilliant green agar (BGA) plates containing C–N.

2.8. CpG-ODN treatments and SE organ invasion

Each experiment consisted of seven groups with 20 chickens per group: Control (SE challenging group receiving no CpG treatment), 25 μg CpG-ODN#1/chicken, 50 μg CpG-ODN#1/chicken, 25 μg CpG-ODN#17/chicken, 50 μg CpG-ODN#17/chicken, and 50 μg nCpG-ODN/chicken. Treatments (0.5 ml) of ODNs or PBS (for control group) were given by i.p. injection to newly hatched chickens. Twenty-four hours after ODN treatments, 0.5 ml of SE (5×10^7 CFU/chicken) was orally gavaged to each chicken.

Twenty-four hours after the SE challenge, chickens were euthanized with CO_2. Liver and spleen were aseptically removed from each chicken and cultured as a combined sample in an enrichment tetrathionate broth over night (18–24 h) at 41 °C according to the National Poultry Improvement Plan guidelines [25]. After incubation, the broth was streaked on BGA plates containing C–N and incubated for an additional 24 h at 41 °C. The plates were examined for the presence of C–N resistant SE colonies. SE colonies were confirmed by appropriate antiserum test (DIFCO Lab, Detroit, MI, USA). Four independent experiments were conducted at different date and total of 80 chickens were used for each treatment group.

2.9. CpG-ODN treatments and peritoneal SE infection

Administration of SE via i.p. route has been used to induce a systemic infection in young chicken [26]. In this experiment, newly hatched chickens (20 chickens per treatment group) were i.p. treated with CpG-ODN as described above. Four hours after CpG-ODN treatments, chickens were given by i.p. injection either 0.1 ml sterile PBS or 0.1 ml 5×10^4 CFU/ml of SE (5×10^3 CFU/chicken). The dosage was previously determined by a pilot experiment to induce at least 50% mortality in day-old chickens. After challenge, experimental chickens were monitored twice daily and dead chickens were removed for the duration of seven days. Four independent experiments were conducted at different date and total of 80 chickens were used for each treatment group.

2.10. Data analysis

Data from four independent experiments were statistically analyzed by one way ANOVA followed by

multiple comparisons (Tukey test) of treatments versus control group using SigmaStat® software (Jandel Scientific, San Rafael, CA, USA). $p = 0.05$ was considered statistically significant.

3. Results

Heterophils and PBMC from neonatal chickens were used to evaluate innate immune responses to CpG-ODN by measuring cell degranulation, oxidative burst, and induction of NO. The degranulation response to CpG-ODN stimulation was measured only in heterophils. Since heterophils produce little NO when activated with microbial agonist, such as lipopolysaccharide (data not shown), the induction of NO by CpG-ODN was measured only in monocytes.

3.1. Induction of heterophil degranulation by CpG-ODN

Stimulation of chicken heterophils with CpG-ODN induced degranulation activity. Significantly higher β-glucuronidase activities were obtained in the culture media of heterophils treated with CpG-ODNs (Fig. 1). The degranulation activity of heterophils in response to CpG-ODN stimulation was comparable with cells stimulated by formalin-killed SE (FKSE was prepared by incubation of bacteria in 1% formalin solution at 4 °C for 24 h followed by washing four times with PBS to remove the formalin). CpG-ODNs were more effective than the control ODN (nCpG-ODN) in induction of heterophil degranulation. However, increased degranulation was observed in heterophils stimulated with nCpG-ODN. This indicated certain degree of none-CpG-motif-specific responses to ODN stimulations.

3.2. Induction of oxidative burst in heterophils and monocytes by CpG-ODN

Oxidative burst that generates ROS is an innate immune response of immune cells, such as heterophils and monocytes/macrophage, to microbes and microbial components. Significant increase of oxidative burst was observed when heterophils or monocytes were stimulated with ODNs (Fig. 2). However, the response of oxidative burst was not CpG motif specific, since nCpG-ODN also induced oxidative burst in both heterophils and monocytes.

3.3. Induction of NO production in monocytes by CpG-ODN

Induction of NO synthesis in chicken monocytes after stimulation with 10 μg/ml of CpG-ODN and nCpG-ODN was measured after 48 h incubation at 41 °C (Fig. 3). The induction of NO in chicken monocytes was CpG motif sequence-dependent, with CpG-ODN#17 containing motif GTCGTT strongly stimulated NO production; while CpG#1induced significantly less NO production and nCpG-ODN did not stimulate any NO production. CpG-ODN#17 seems to be more potent in induction of NO synthesis in monocytes than a well-known microbial agonist LPS. These results indicated sequence-specific recognition of CpG-ODN by chicken monocytes in induction of NO synthesis.

3.4. Reduction of SE organ invasion in neonatal chicken by i.p. CpG-ODN

Effects of i.p. injection of CpG-ODNs on the incidence of SE organ invasion induced by oral challenge in neonatal chickens were evaluated (Table 1). CpG-ODN#17 treatments reduced SE organ invasion in a

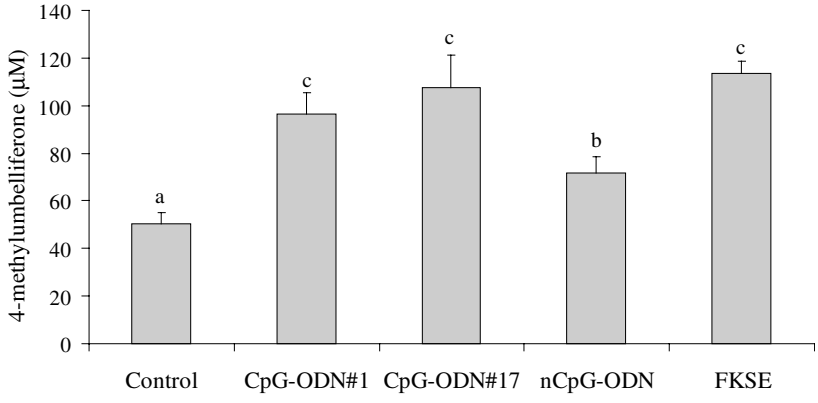

Fig. 1. Heterophil degranulation induced by stimulation of CpG-ODN, nCpG-ODN, and formalin-killed SE whole bacterial (FKSE, as control). Data are mean and SD of pooled data from tow independent experiments. Heterophils were isolated from pooled peripheral of 100 chickens in each experiment and at least five replicates assays were run for each treatment. Data with different letter indicate that the difference is statistically significant ($p \leqslant 0.05$).

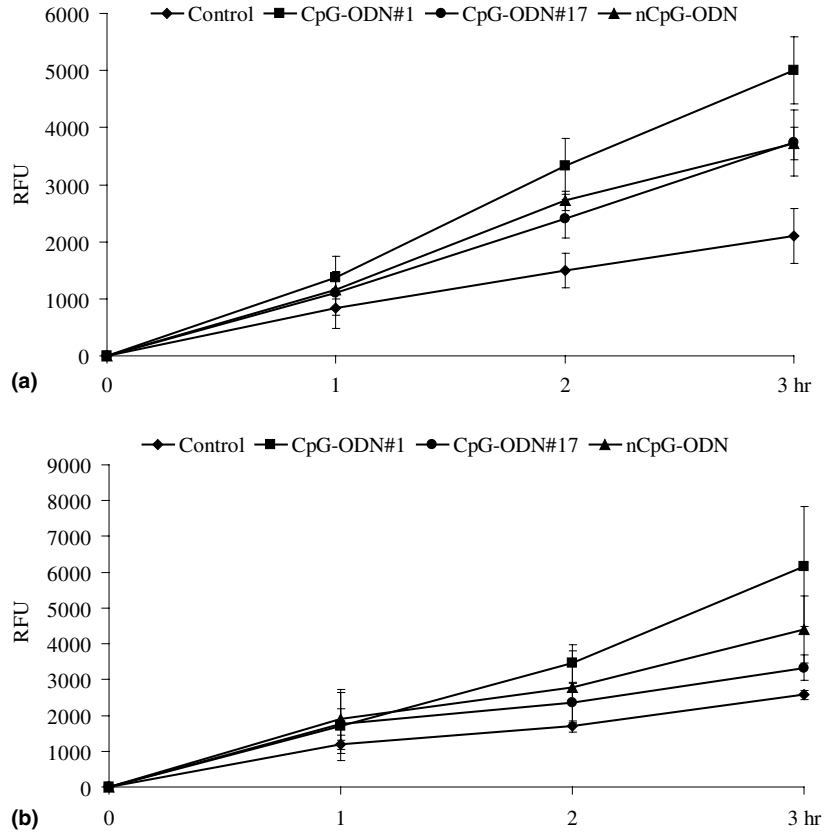

Fig. 2. Heterophils (a) and PBMC (b) oxidative burst stimulated by ODNs measured as an arbitrary relative fluorescent unit (RFU) increase during the oxidation of DCFH-DA to fluorescent DCF. Data are mean and SD of pooled data from tow independent experiments. Heterophils and PBMC were isolated from peripheral blood of 100 chickens in each experiment and at least five replicates assays were run for each treatment.

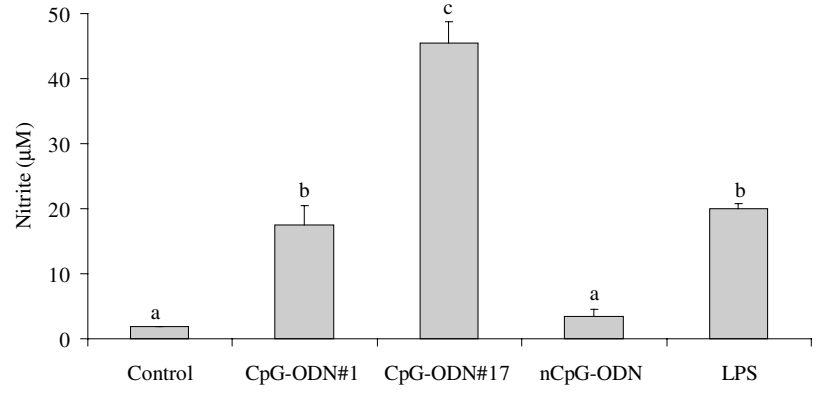

Fig. 3. NO production measured as nitrite accumulated in cell cultural media of chicken peripheral blood monocytes after stimulation with ODN and LPS (as positive control) for 48 h. Data are mean and SD of pooled data from tow independent experiments. Heterophils and PBMC were isolated from peripheral blood of 100 chickens in each experiment and at least five replicates assays were run for each treatment. Data with different letter indicate that the difference is statistically significant ($p \leqslant 0.05$).

dosage-dependent manner. A marginal reduction ($p = 0.123$) of SE organ invasion (26.2%) was observed on chickens receiving 25 μg CpG-ODN#17/chicken. However, increasing CpG-ODN#17 to 50 μg/chicken reduced SE organ invasion to a significantly lower level ($p < 0.001$) of 5.1% compared to 42.5% in the control group. On the other hand, a relatively higher rate of SE organ colonization (65%) was observed on chickens receiving 25 μg CpG-ODN#1/chicken. A moderate reduction ($p = 0.114$) of SE organ invasion (21.3%) was achieved when chickens treated with 50 μg CpG-ODN#1/chicken. The reason for the high incidence of SE organ invasion in the group treated with 25 μg CpG-ODN#1/chicken cannot be readily explained.

Table 1
Effects of CpG-ODN treatments on *Salmonella enteritidis* organ invasion in neonatal chickens

Treatments[b]	SE(+)%[a]				Mean ± SEM[c]
	Experiment 1	Experiment 2	Experiment 3	Experiment 4	
Control (PBS)	40	35	40	55	43 ± 4
CpG-ODN#1 (25 μg)	75	80	70	35	65 ± 10
CpG-ODN#1 (50 μg)	15	20	40	10	21 ± 7
CpG-ODN#17 (25 μg)	16	44	35	10	26 ± 8
CpG-ODN#17 (50 μg)	0	11	5	5	5 ± 2*
nCpG-ODN#2 (50 μg)	50	45	35	40	43 ± 3

[a] Data represent four independent experiments conducted at different date. SE(+)% = 100 × [SE(+) chickens/Survival chickens].
[b] Each treatment group had 20 chickens in each experiment.
[c] Means with (*) indicate the significant difference ($p < 0.05$) compared to the control group determined by Tuckey test.

Table 2
Effects of CpG-ODN treatments on *Salmonella enteritidis* peritoneal infection induced mortality in neonatal chickens

Treatments[b]	Accumulated mortality (%)[a]		
	Day-3	Day-5	Day-7
Control (PBS)	45 ± 5	65 ± 5	78 ± 8
CpG-ODN#1 (25 μg/bird)	23 ± 1	40 ± 3	51 ± 3
CpG-ODN#1 (50 μg/bird)	39 ± 7	58 ± 5	61 ± 6
CpG-ODN#17 (25 μg/bird)	19 ± 2*	35 ± 6*	40 ± 5*
CpG-ODN#17 (50 μg/bird)	9 ± 6*	33 ± 9*	45 ± 8*
nCpG-ODN#2 (50 μg/bird)	40 ± 5	53 ± 7	65 ± 5

[a] Data represent means and SEM from four independent experiments conducted at different date. Means with (*) indicate the significant difference ($p < 0.05$) compared to the control group determined by Tuckey test.
[b] Each treatment group had initial 20 chickens in each experiment.

Chickens receiving the nCpG-ODN showed no difference ($p = 1.000$) in the SE organ invasion (42.5%) from the challenge group.

3.5. Reduction of peritoneal SE infection induced mortality in neonatal chicken by i.p. CpG-ODN

Acute peritoneal infection of SE induced significant mortality in neonatal chickens during the period of seven day experiment. Effects of i.p. CpG-ODN on SE peritoneal infection induced mortality in neonatal chickens varied depending on type of CpG-ODN. Significant reduction of mortality was observed on chickens treated with i.p. CpG-ODN#17 at both dosage of 25 and 50 μg/chicken (Table 2). The protective effects of CpG-ODN#17 were demonstrated throughout the seven-day-period of experiment, whereas, CpG-ODN#1and nCpG-ODN have shown no protective effect on SE induced mortality.

4. Discussion

Innate immune system has developed and conserved during the course of evolution for quickly recognizing and responding to invading pathogens through recognition of pathogen associated molecule patterns (PAMPs). One example of such recognition of PAMPs is that vertebrate innate immune system (from fish, birds to mammals) can discriminate bacterial DNA from self DNA by recognizing unmethylated CpG motif presented in the bacterial DNA. These immune stimulatory activities of CpG-ODN have been studied extensively and well characterized in human and murine immune cells. However, responses of avian immune system to bacterial DNA or CpG-ODN are largely unknown. Here, we have demonstrated in vitro activation of chicken leukocytes, heterophils and PBMC, and in vivo protection of neonatal chickens from SE organ invasion and mortality induced by peritoneal SE infection by CpG-ODN.

Our study has clearly demonstrated immune recognition of CpG-ODN by chicken heterophils and monocytes. This recognition of CpG-ODN leaded to activation of effector functions in heterophils. One of those functions is degranulation, the process of mobilization and release of heterophil granules containing bactericidal substances including cationic bactericidal peptides [27], defensins [28], and proteolytic enzyme such as gelatinase B [29]. These granule proteins are pre-packaged in the granules and released in response to certain microbial agonist stimulation to engage bactericidal activities.

In additional to degranulation, CpG-ODN also stimulated generation of ROS in both heterophils and monocytes. ROS production is mediated by a multicomponent enzyme complex, NADPH oxidase [30]. The

superoxide anion (O_2^-) is first produced and then converted to hydrogen peroxide (H_2O_2) with either spontaneous dismutation itself or with facilitation of superoxide dismutase (SOD). H_2O_2, in the presence of transition metals such as iron or copper, can give rise to an extremely reactive and toxic hydroxyl radical ($OH^{\cdot}$). ROS generation (oxidative burst) in response to microbe and microbial component stimulation has been previously studied mostly in heterophils [23,31–34]. However, it was the first time to demonstrate that CpG-ODN stimulates oxidative burst in chicken heterophils and monocytes. Results from this study indicated that induction of oxidative burst by CpG-ODN in heterophils and monocytes was not related to CpG sequence, since the nCpG-ODN which does not contain CpG sequence also stimulated oxidative burst. Therefore, this particular immune stimulatory activity of CpG-ODN may not be mediated by the TLR9. Receptor recognizing ODN and mediating oxidative burst remains to be determined.

NO mediates a number of biological reactions ranging from endothelium-dependent relaxation to macrophage antimicrobial and tumoricidal activity [35]. NO has been shown to contribute to host defense against intracellular pathogenic microorganisms such as *Salmonella* [35,36] and to inhibit virus proliferation [36,37]. In a recent study, in vivo blockage of NO production by an inhibitor also blocked host defense to *Salmonella* as evidenced by high mortality and high level of bacteria colonization in liver and spleen [35]. Previously, our study [11] demonstrated that CpG-ODNs were able to activate an avian macrophage cell line and induce NO production and cytokine expression. In this study, our results further demonstrated that CpG-ODN also stimulated NO synthesis in primary PBMC from the neonatal chicken. It seems that chicken PBMC recognized CpG-ODN and readily induced NO synthesis without prerequisite of priming as reported on mammalian macrophage cells [38,39]. The stimulation activity of CpG-ODN was comparable or exceeded to a well-known microbial agonist LPS. Results from above in vitro studies suggest that CpG-ODN can activate innate immune functions of neonatal chicken immune cells. Different mechanisms may be involved in recognition of CpG-ODN and activation of certain immune function.

In addition to in vitro immune stimulatory activity of CpG-ODN, experiments were conducted to determine the in vivo protective function by evaluating effects of i.p. administrated CpG-ODN on SE organ invasion and mortality induced by peritoneal SE infection. Our results have demonstrated that i.p. injection of CpG-ODN#17, which previously has been shown to strongly stimulate NO production and IL-1β expression in HD11 avian macrophage cell line [11] and confirmed immune stimulatory activities on primary heterophils and PBMC in this study, significantly reduced the incidence of SE organ invasion in neonatal chickens. CpG-ODN#17 treatments demonstrated dose-dependent reduction of SE organ invasion. Chickens receiving 50 μg of CpG-ODN#17 had only 5.1% SE positive in liver and spleen compared to 42.5% in the control group.

Similarly, i.p. administered CpG-ODN#17 significantly reduced mortality rate of the experimental chickens with peritoneal SE infection. Neither CpG-ODN#1 nor nCpG-ODN had effects on peritoneal SE infection induced mortality in this study. Results from both SE organ invasion and peritoneal SE induced mortality demonstrated immune protection against SE infection by CpG-ODN#17. These results also indicated that activation of heterophils and macrophage primed innate immunity for more efficient responses to eliminate or reduce infectious pathogen at early stage of infection. However, underline mechanism of CpG-ODN induced protection against SE infection observed from this study has not been defined and need to be further studied. On the other hand, CpG-ODN has been shown to protect mice from infections by intracellular bacteria and parasites [17–20]. An immune stimulatory CpG-ODN (2006) for human immune cells [40] was recently found to increase intracellular killing of SE in HD11 avian macrophage cell line [14]. In an another study, subcutaneous injection of CpG (sequence not given) was reported to prevent both mortality and development of cellulites in three week old chicken from *E. coli* infection [13]. Our study has provided further evidence that immune stimulatory CpG-ODN modulates innate immunity to increase resistance to infectious diseases in poultry.

Acknowledgements

Authors wish to thank Laura Ripley for her assistance in necropsy. Mention of commercial or proprietary products in this paper does not constitute an endorsement of these products by the USDA, nor does it imply the recommendation of products by the USDA to the exclusion of similar products.

References

[1] Krieg, A.M. (2002) CpG motifs in bacterial DNA and their immune effects. Ann. Rev. Immunol. 20, 709–760.
[2] Bird, A.P. (1987) CpG islands as gene markers in the vertebrate nucleus. Trends Genet. 3, 342–347.
[3] Krieg, A.M., Yi, A., Matson, S., Waldschmidt, T.J., Bishop, G.A., Teasdale, R., Koretzky, G.A. and Klinman, D.M. (1995) CpG motifs in bacterial DNA trigger direct B-cell activation. Nature 374, 546–549.
[4] Yamamoto, S., Yamamoto, T., Kataoka, T., Kuramoto, E., Yano, O. and Tokunaga, T. (1992) Unique palindromic sequences in synthetic oligonucleotides are required to induce IFN abd

augment IFN-mediated natural killer activity. J. Immunol. 148, 4072–4076.
[5] Hemmi, H., Takeuchi, O., Kawai, T., Kaisho, T., Sato, S., Sanjo, H., Matsumoto, M., Hoshino, K., Wagner, H., Takeda, K. and Akira, S. (2000) A Toll-like receptor recognizes bacterial DNA. Nature 408, 740–745.
[6] Bauer, S., Kirschning, C.J., Häcker, H., Redecke, V., Hausmann, S., Akira, S., Wagner, H. and Lipford, G.B. (2001) Human TLR9 confers responsiveness to bacterial DNA via species-specific CpG motif recognition. Proc. Natl. Acad. Sci. USA 98, 9237–9242.
[7] Jorgensen, J.B., Johansen, A., Stenersen, B. and Sommer, A.I. (2001) CpG oligodeoxynucleotides and plasmid DNA stimulate Atlantic salmon (*Salmo salar* L.) leucocytes to produce supernatants with antiviral activity. Dev. Comp. Immunol. 25, 313–321.
[8] Jorgensen, J.B., Zou, J., Johansen, A. and Secombes, C.J. (2001) Immunostimulatory CpG oligodeoxynucleotides stimulate expression of IL-1beta and interferon-α like cytokines in rainbow trout macrophages via a chloroquine-sensitive mechanism. Fish Shellfish Immunol. 11, 673–682.
[9] Meng, Z., Shao, J. and Xiang, L. (2003) CpG oligodeoxynucleotides activate grass carp (*Ctenopharyngodon idellus*) macrophages. Dev. Comp. Immunol. 27, 313–321.
[10] Vleugels, B., Ververken, C. and Goddeeris, B.M. (2002) Stimulatory effect of CpG sequences on humanoral responses in chickens. Poult. Sci. 81, 1317–1321.
[11] He, H., Crippen, T.L., Farnell, M.B. and Kogut, M.H. (2003) Identification of CpG oligodeoxynucleotide motifs that stimulate nitric oxide and cytokine production in avian macrophage and peripheral blood mononuclear cells. Dev. Comp. Immunol. 27, 621–627.
[12] He, H. and Kogut, M.H. (2003) CpG-ODN-induced nitric oxide production is mediated through Clathrin-dependent endocytosis, endosomal maturation, and activation of PKC, MEK1/2 and p38 MAPK, and NF-κB pathways in avian macrophage cells (HD11). Cell Signal. 15, 911–917.
[13] Babiuk, L.A., Gomis, S. and Hecker, R. (2003) Molecular approaches to disease control. Poult. Sci. 82, 870–875.
[14] Xie, H., Raybourne, R.B., Babu, U.S., Lillehoj, H.S. and Heckert, R.A. (2003) CpG-induced immunomodulation and intracellular bacterial killing in a chicken macrophage cell line. Dev. Comp. Immunol. 27, 823–834.
[15] Janeway Jr., C.A. and Medzhitov, R. (2002) Innate immune recognition. Ann. Rev. Immunol. 20, 197–216.
[16] Well, L.L., Lowry, V.K., DeLoach, J.R. and Kogut, M.H. (1998) Age-dependent phagocytosis and bactericidal activities of the chicken heterophil. Dev. Comp. Immunol. 22, 103–109.
[17] Krieg, A.M., Love-Homan, L., Yi, A.K. and Harty, J.T. (1998) CpG DNA induces sustained IL-12 expression in vivo and resistance to *Listeria monocytogenes* challenge. J. Immunol. 161, 2428–2434.
[18] Klinman, D.M., Conover, J. and Coban, C. (1999) Repeated administration of synthetic oligodeoxynucleotides expressing CpG motifs provides long-term protection against bacterial infection. Infect. Immun. 67, 5658–5663.
[19] Zimmermann, S., Egeter, O., Hausmann, S., Lipford, G.B., Rocken, M., Wagner, H. and Heeg, K. (1998) CpG oligodeoxynucleotides trigger protective and curative Th1 responses in lethal murine leishmaniasis. J. Immunol. 160, 3627–3630.
[20] Gramzinski, R.A., Doolan, D.L., Sedegah, M., Davis, H.L., Krieg, A.M. and Hoffman, S.L. (2001) Interleukin-12- and γ interferon-dependent protection against malaria conferred by CpG oligodeoxynucleotide in mice. Infect. Immun. 69, 1643–1649.
[21] National Research Council (1994) Nutrient Requirements of Poultry, 9th ed. Washington DC: Academy Press.
[22] Kogut, M.H, Genovese, K.J. and Lowry, V.K. (2001) Differential activation of signal transduction pathways mediated phagocytosis, oxidative burst, and degranulation by chicken heterophils in response to stimulation with opsonized *Salmonella enteritidis*. Inflammation 25, 7–15.
[23] He, H., Farnell, M.B. and Kogut, M.H. (2003) Inflammatory agonist stimulation and signal pathway of oxidative burst in neonatal chicken heterophils. Comp. Biochem. Physiol. A 135, 177–184.
[24] Green, L., Wagner, D., Glogowski, J., Skipper, P., Wishnok, J. and Tannenbaum, S. (1982) Analysis of nitrate, nitrite and [15N] nitrate in biological fluids. Analyt. Biochem. 126, 131–138.
[25] United State Department of Agriculture, Animal and Plant Health Inspection Service (1989) National Poultry Improvement Plan and Auxiliary Provision, Veterinary Services, Publication APHIS, pp. 40–91. US Government Printing Office, Washington DC.
[26] Kogut, M.H., McGruder, E.D., Hargis, B.M., Corrier, D.E. and Deloach, J.R. (1995) Characterization of the pattern of inflammatory cell influx in chicks following the intraperitoneal administration of live *Salmonella enteritidis* and *Salmonella enteritidis*-immune lymphokines. Poult. Sci. 74, 8–17.
[27] Evans, E.W., Beach, F.G., Moore, K.M., Jackwood, M.W., Glisson, J.R. and Harmon, B.G. (1995) Antimicrobial activity of chicken and turkey heterophil peptides CHP1, CHP2, THP1, and THP3. Vet. Microbiol. 47, 295–303.
[28] Brockus, C.W., Jackwood, M.W. and Harmon, B.G. (1998) Characterization of β-defensin prepropeptide mRNA from chicken and turkey bone marrow. Anim. Genet. 29, 283–289.
[29] Hahn-Dantona, E.A., Aimes, R.T. and Quigley, J.P. (2000) The isolation, characterization, and molecular cloning of a 75-kDa gelatinase B-like enzyme, a member of the matrix metalloproteinase (MMP) family. An avian enzyme that is MMP-9-like in its cell expression pattern but diverges from mammalian gelatinase B in sequence and biochemical properties. J. Biol. Chem. 275, 40827–40838.
[30] Thannckal, V.J. and Fanburg, B.L. (2000) Reactive oxygen species in cell signaling. Am. J. Physiol. Lung Cell Mol. Physiol. 279, L1005–L1028.
[31] Kogut, M.H., Genovese, K.J. and Lowry, V.K. (2001) Differential activation of signal transduction pathways mediated phagocytosis, oxidative burst, and degranulation by chicken heterophils in response to stimulation with opsonized *Salmonella enteritidis*. Inflammation 25, 7–15.
[32] Kogut, M.H., Genovese, K.J. and Nisbet, D.J. (2001) Signal transduction pathways activated by engaging immunoglobulin Fc receptors on chicken heterophils. Dev. Comp. Immunol. 25, 639–646.
[33] Farnell, M.B., He, H. and Kogut, M.H. (2003) Differential activation of signal transduction pathways mediating oxidative burst by chicken heterophils in response to stimulation with lipopolysaccharide and lipoteichoic acid. Inflammation 27, 225–231.
[34] Farnell, M.B., He, H., Genovese, K.J. and Kogut, M.H. (2003) Pharmacological analysis of signal transduction pathways required for oxidative burst in chicken heterophils stimulated by a Toll-like receptor 2 agonist. Int. Immunopharmacol. 3, 1677–1684.
[35] Eisenstein, T.K. (2001) Implications of *Salmonella*-induced nitric oxide (NO) for host defense and vaccines: NO, an antimicrobial, antitumor, immunosuppressive and immunoregulatory molecule. Microbes Infec. 3, 1223–1231.
[36] Nathan, C. and Shiloh, M.U. (2000) Reactive oxygen and nitrogen intermediates in the relationship between hosts and microbial pathogens. Proc. Natl. Acad. Sci. USA 97, 8841–8848.
[37] Djeraba, A., Musset, E., Bernardet, N., Vern, Y.L. and Quéré, P. (2002) Similar pattern of iNOS expression, NO production and

cytokine response in genetic and vaccination-aquired resistance to Marek's disease. Vet. Immunol. Immunopathol. 85, 63–75.

[38] Sweet, M.J., Stacey, K.J., Kakuda, D.K., Markovich, D. and Hume, D.A. (1998) IFN-gamma primes macrophage responses to bacterial DNA. J. Interferon Cytokine Res. 18, 263–271.

[39] Gao, J.J., Zuvanich, E.G., Xue, Q., Horn, D.L., Silverstein, R. and Morrison, D.C. (1999) Cutting edge: bacterial DNA and LPS act in synergy in inducing nitric oxide production in RAW 264.7 macrophages. J. Immunol. 163, 4095–4099.

[40] Hartmann, G., Weeratna, R.D., Ballas, Z.K., Payette, P., Blackwell, S., Suparto, I., Rasmussen, W.L., Waldschmidt, M., Sajuthi, D., Purcell, R.H., Davis, H.L. and Krieg, A.M. (2000) Delineation of CpG phosphorothioate oligodeoxynucleotide for activating primate immune responses in vitro and in vivo. J. Immunol. 164, 1617–1624.